Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 427–432, January 1996
Neurobiology

Neuroprotective strategy for Alzheimer disease: Intranasal administration of a fatty neuropeptide

I. GOZES*†, A. BARDEA*, A. RESHEF*, R. ZAMOSTIANO*, S. ZHUKOVSKY*, S. RUBINRAUT‡, M. FRIDKIN‡, AND D. E. BRENNEMAN§

*Department of Clinical Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel; ‡Department of Organic Chemistry, Weizmann Institute of Science, Rehovot 76100, Israel; and §Section on Developmental and Molecular Pharmacology, Laboratory of Developmental Neurobiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892

Communicated by Viktor Mutt, Karolinska Institute, Stockholm, Sweden, October 17, 1995

ABSTRACT **Neurodegenerative diseases, in which neuronal cells disintegrate, bring about deteriorations in cognitive functions as is evidenced in millions of Alzheimer patients. A major neuropeptide, vasoactive intestinal peptide (VIP), has been shown to be neuroprotective and to play an important role in the acquisition of learning and memory. A potent lipophilic analogue to VIP now has been synthesized, [stearyl-norleucine^{17}]VIP ([St-Nle^{17}]VIP), that exhibited neuroprotection in model systems related to Alzheimer disease. The β-amyloid peptide is a major component of the cerebral amyloid plaque in Alzheimer disease and has been shown to be neurotoxic. We have found a 70% loss in the number of neurons in rat cerebral cortical cultures treated with the β-amyloid peptide (amino acids 25–35) in comparison to controls. This cell death was completely prevented by cotreatment with 0.1 pM [St-Nle^{17}]VIP. Furthermore, characteristic deficiencies in Alzheimer disease result from death of cholinergic neurons. Rats treated with a cholinergic blocker (ethylcholine aziridium) have been used as a model for cholinergic deficits. St-Nle-VIP injected intracerebroventricularly or delivered intranasally prevented impairments in spatial learning and memory associated with cholinergic blockade. These studies suggest both an unusual therapeutic strategy for treatment of Alzheimer deficiencies and a means for noninvasive peptide administration to the brain.**

Senile dementia of the Alzheimer type afflicts 3–5 million people in the United States (1, 2). Although the etiology of this disease remains unclear, recent studies have indicated that the β-amyloid peptide is excessively deposited in the brains of the patients, contributing to the neurodegenerative process, which leads to senility (3–5). A major class of neurons that are known to be lost in Alzheimer disease are cholinergic neurons (6–8). Cholinergic blockade, resulting in impairment of learning and memory, has been used as a model of this disease (9). Ethylcholine aziridium (AF64A) is a blocker of choline uptake and it is well established that intracerebroventricular (i.c.v.) administration of this drug can induce loss in cholinergic neurons at the basal forebrain (9–11). Despite this apparent progress, successful treatment of neurodegeneration associated with Alzheimer dementia remains elusive (1, 2, 12, 13).

In the present study, we have explored the possibility that a neurotrophic peptide, vasoactive intestinal peptide (VIP) (14–16), might provide neuroprotection in models of degeneration/cognitive impairment related to Alzheimer disease. This strategy is based on the demonstrated ability of VIP to protect neurons in the central nervous system from a variety of neurotoxic substances including tetrodotoxin (17) and the human immunodeficiency virus (HIV) envelope protein (18), suggesting that this peptide can provide general neuroprotection. Numerous anatomical studies combining immunohistochemistry, radioimmunoassays, receptor distribution (as reviewed in refs. 14 and 15), and gene expression studies (19–21) attest to the availability of VIP in brain areas important for cognitive functions, such as the cerebral cortex and the hippocampus. However, VIP expression is significantly decreased in the cerebral cortex of aged animals (22). Blockade of VIP actions by a specific antagonist (23) or experimentally reduced VIP expression in transgenic animals (24) both resulted in behavioral deficits associated with impairment of learning and memory. Furthermore, VIP may modulate brain activity through its ability to enhance cholinergic function (25–28). Although there is conflicting evidence concerning the role of VIP in dementia in general and in Alzheimer disease in particular, a study of immunoreactivity of VIP in Alzheimer and control brains showed a significant reduction of VIP immunoreactivity in the cerebral cortex, especially in the insular and angulate cortex of Alzheimer patients (29). However, it has never been determined whether this reduction was the cause or the result of deterioration of the cortex. The present work clearly indicated that a new lipophilic VIP analogue (30–32) provided potent and effective protection from neuronal cell death produced by β-amyloid peptide *in vitro* and from impairment of spatial learning produced by cholinergic blockade *in vivo*.

MATERIALS AND METHODS

Peptide Syntheses. Peptide syntheses were carried out manually according to the solid-phase strategy employing optimum side chain protection as before (30–32). Products were purified by gel chromatography on a Sephadex G-25 column, followed by reversed-phase HPLC on a semipreparative C_8 column (Lichrosorb RP-8) (7 μm; 250 × 10 mm; Merck). Elution of peptides was affected by linear gradients established between 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in 75% (vol/vol) acetonitrile in water. The pure peptides showed the desired molar ratios of the constituent amino acids. Molecular weights were ascertained by mass spectroscopy (VG Tofspec, Laser Desorption mass spectrometer, Fison Instruments, Loughborough, England). Sequences were determined with a gas phase Applied Biosystems model 470A protein microsequencer coupled to Applied Biosystems model 120A PTH analyzer.

Cell Culture. Cerebral cortical cell cultures were prepared by a slight modification of the techniques described by Forsythe and Westbrook (33), in which cerebral cortex was used instead of hippocampus and newborn rats were used instead of embryonic day 16 mice. Cerebral cortical neurons (1.5×10^5 cells per 35-mm dish) were plated on confluent cerebral cortical astrocyte feeder cultures as described (34). The culture

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: i.c.v., intracerebroventricular(ly); VIP, vasoactive intestinal peptide; [St-Nle^{17}]VIP, [stearyl-norleucine^{17}]VIP.

†To whom reprint requests should be addressed.

medium was modified Eagle's medium containing 5% horse serum and N3 [medium supplement containing a hormone mixture, according to Romijn *et al.* (35)]. After 9 days growth *in vitro*, the cultures were given a complete change of medium and treated with the appropriate peptide for 5 additional days. [Stearyl-norleucine[17]]VIP ([St-Nle[17]]VIP) (10^{-3} M) was dissolved in absolute ethanol and serially diluted in distilled sterile phosphate-buffered saline. Fifteen microliters of the test peptide was then added to 1.5 ml of tissue culture medium to yield the final desired concentration. Under these conditions, the highest percentage of ethanol used was 0.001%. At this concentration and even at 0.01% ethanol, there was no effect on neuronal survival. In the case of VIP 0.01 M acetic acid was used instead of ethanol (17, 18). The astrocyte feeder layer contained confluent, dividing cells at days 9 and 14. Cell division was determined by thymidine incorporation performed as described (36) and astroglial cell content was evaluated by specific immunostaining with glial fibrillar acidic protein antibodies and iodinated secondary antibodies. Results have indicated no apparent change between β-amyloid-treated and untreated cultures. After 14 days in culture, cells were fixed for immunocytochemistry and stained with antibodies against neuron-specific enolase (a neuronal marker). Neuronal cell counts were executed on 40 fields, with a total area of 26 mm^2. Neurons were counted without knowledge of type of treatment as described (e.g., see refs. 17 and 18). Experiments were performed at least four times, each in triplicate. There was no effect of vehicle treatment on the cell counts in all experimental paradigms.

***In Vivo* Model for Cholinergic Inhibition.** Male rats (either Charles River Breeding Laboratory or Wistar; 250–300 g) were injected i.c.v. at a rate of 0.21 μl/min, using plastic tubing (PE-20) attached to a 25-gauge needle; controls received an injection of saline (2 μl per side), and experimental animals received injections of the cholinergic blocker AF64A (3 nmol per 2 μl per side; see refs. 9–11). In one set of experiments, animals were stereotaxically implanted during injection to allow drug application under 10% chloral hydrate anesthesia. The cannula was a stainless steel needle (21 gauge; Becton Dickinson—i.e., a cannula of a 21G needle was attached to the 25G needle used for the AF64A injection). The cannula was placed 1 mm posterior and 1–1.5 mm lateral to bregma, 3.5 mm below the surface of the cranium. Only one brain side was cannulated (the left side). Four stainless steel screws were placed around the cannula and acrylic dental cement was used to anchor the cannula. Placement in the lateral ventricle was subsequently confirmed by injection of dye.

Drug Administration and Behavioral Testing. Drug treatment was initiated 7–10 days after cannulation in animals that were injected with AF64A and cannulated. These animals were divided into two equal groups and were thereafter injected daily (i.c.v.), one group with saline (4 μl per animal; control rats) and the other with 100 ng per 4 μl of [St-Nle[17]]VIP for each animal. The control rats were chronically injected with saline through the cannula.

In a second set of experiments, animals treated with AF64A but not cannulated received daily nasal administration of [St-Nle[17]]VIP dissolved in 10% sefsol and 40% isopropanol (32) at a concentration of either 10 μg or 70 μg per 40 μl (20 μl administered through each nostril). Control animals (those treated with saline at the time of injection of the experimental groups with AF64A) received intranasal administration of either the vehicle or [St-Nle[17]]VIP. The rats were partially anesthetized by diethyl ether prior to nasal administration. After 7 days of drug administration, behavioral assays were conducted for an additional 11 days. Drugs were injected i.c.v. 4 hr prior to behavioral testing or applied by nasal administration 1 hr prior to testing. All animals were daily treated with 50,000 units of durabiotic antibiotics to avoid infection.

For behavioral test procedures, rats were placed in a circular pool, 1.26 m in diameter, 0.2 m deep, equipped with a clear Plexiglas column, with a 13.3-cm platform reaching just below the surface of the water (22–24°C). The latency of reaching the platform was recorded for each rat (in seconds) and the decreases over days of training reflected learning and memory (23, 24, 37–39). Rats were tested once daily. The cutoff time for the latency to reach the platform was 300 sec. If the animal did not find the platform after 300 sec, it was then placed on the platform for 20 sec. Two types of experiments were performed. In one type of experiment, the platform was set in the middle of the pool. In another type of experiment, the platform was set in the middle of one quadrant of the pool. Each day (each trial) the animals were started from a different start point. If we consider the pool as a clock, then the animals were placed at hour positions 6:00, 9:00, 12:00, and 3:00 repeatedly, in the same order. In some experiments, to avoid bias related to possible changes in motor activity in the various treatment groups, a probe trial that assessed spatial memory was also utilized as follows. After 9 days of training and testing, the platform was removed and on day 10 the animals were subjected to swimming in the pool (for 300 sec) without the platform; in these experiments, the time spent by the rats in the quadrant of the pool where the platform used to be was recorded (39). To evaluate for possible motor deficits that could impair performance, unrelated to memory, in some experiments, on day 11 of testing the animals were allowed to swim to an exposed platform.

Biodistribution Following Intranasal Administration. [St-Nle[17]]VIP was radioiodinated as described (30, 32) and $\approx 3 \times 10^6$ cpm per 2 μl per rat was applied intranasally to rats (250–300 g). Animals were sacrificed 15 min after drug administration and tissue samples were weighed and assayed for radioactivity in a γ-counter. Radioactive tissue samples (e.g., brainstem containing 150 cpm per sample) were thereafter homogenized and subjected to centrifugation ($5400 \times g$ for 25 min). Supernatants were then subjected to HPLC analysis (30–32) against [St-Nle[17]]VIP as a marker (eluting with an acetonitrile gradient at fraction 30). Samples were monitored for radioactivity in a γ-counter.

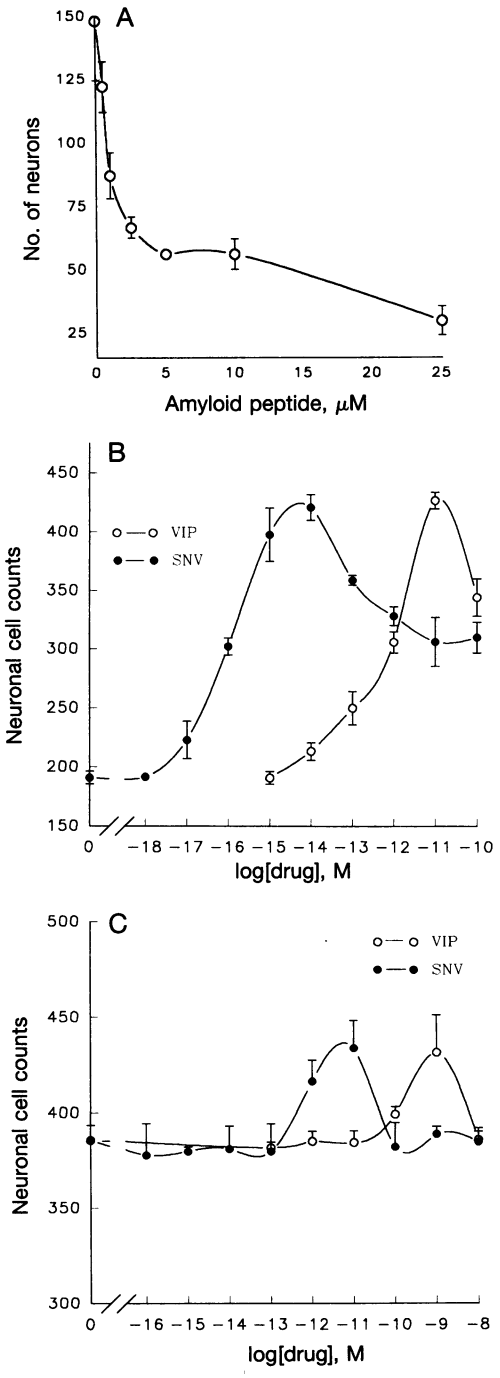
RESULTS

VIP Protects Against Alzheimer-Related Neuronal Cell Death *in Vitro*. To test VIP-related analogues for neuroprotective actions, dissociated cerebral cortical cultures were treated with β-amyloid peptide. This peptide (amino acids 1–40) and some of its fragments have been shown previously to cause neuronal cell death *in vitro* (40, 41). Accordingly, cultures treated (for 5 days) with β-amyloid peptide (amino acids 25–35) exhibited a dose-dependent decrease in neuronal cell counts in comparison to controls (Fig. 1*A*). In multiple experiments, maximal cell death (50–70%) was observed with 25 μM β-amyloid peptide. In control cultures, there was a maximal reduction of 20% in neuronal cell counts between days 9 and 14. Cotreatment with VIP plus β-amyloid peptide prevented neuronal cell death associated with the amyloid peptide [control cultures had 385 ± 8, β-amyloid-treated cultures had 191 ± 5, and β-amyloid + VIP (10^{-11} M)-treated cells had 427 ± 7; $P < 0.05$, one-way analysis of variance, Student–Newman–Keuls method]. The VIP neuroprotective effect was exhibited over a very narrow range of concentrations (Fig. 1*B*). Similar studies with secretin provided no apparent neuroprotective action (data not shown).

A VIP Analogue, [St-Nle[17]]VIP, with Greater Potency and Efficacy than VIP. In an effort to increase the potency and bioavailability of VIP, a lipophilic analogue was devised (32). Composed of a single amino acid substitution (methionine-17 to norleucine-17) and the addition of a fatty acyl moiety (attachment of stearic acid to the N terminus), the new VIP

analogue ([St-Nle^{17}]VIP) exhibited both a greater potency than VIP and specificity for a VIP receptor associated with neuronal survival in dissociated spinal cord cultures (42). [St-Nle^{17}]VIP protected cortical neurons from the toxic effect of the β-amyloid peptide fragment with severalfold greater potency than that observed for VIP (Fig. 1*B*) exhibiting maximal potency at 10^{-14} M ($P < 0.001$, one-way analysis of variance, Student–Newman–Keuls method). Importantly, the neuroprotective action of [St-Nle^{17}]VIP was efficacious over a range of concentrations broader than that of VIP. In cultures not treated with the β-amyloid peptide (Fig. 1*C*), a dose–response curve to [St-Nle^{17}]VIP indicated 20% protection against naturally occurring cell death at a lipophilic peptide concentration of 10^{-11} M and at 10^{-9} M for VIP ($P < 0.05$, one-way analysis of variance, Student–Newman–Keuls method).

[St-Nle^{17}]VIP Protects Against Alzheimer-Related Retardation of Learning and Memory *in Vivo*. In parallel with the *in vitro* experiments, *in vivo* models related to Alzheimer disease were assessed. For evaluation of learning and memory abilities, rats were tested in a Morris water maze. The latency (in seconds) of reaching the submerged platform of a circular water pool was recorded for each rat and the changes over days of training reflected learning and memory. Two potential models for memory retardation were evaluated: i.c.v. injection of (*i*) the β-amyloid peptide (amino acids 1–40) and (*ii*) a cholinergic blocker, the cholinotoxin AF64A (9–11). Wistar rats were injected with Alzheimer peptide (10 μg per 4 μl in 35% acetonitrile/0.1% trifluoroacetic acid) 1 day before initiation of testing. Subsequently, animals were injected daily, 1 hr before behavioral testing. These animals exhibited a 1-day delay in achieving minimal latency in finding the platform (which was fixed in the middle of the pool) compared to animals injected with vehicle alone (data not shown). In contrast, rats treated with the cholinergic blocker exhibited a 9-day delay (Fig. 2) in comparison to controls, indicating a more severe impairment than that observed with the β-amyloid peptide. The ability of [St-Nle^{17}]VIP to improve learning and memory capacities was therefore tested in AF64A-treated animals. Daily i.c.v. injections of [St-Nle^{17}]VIP completely prevented the learning impairment in animals treated with the cholinergic blocker (Fig. 2; after 7 days of training the AF64A-treated animals were significantly different from both control animals and AF64A + [St-Nle^{17}]VIP-treated animals; $P < 0.001$, one-way analysis of variance; furthermore, AF64A+[St-Nle^{17}]VIP-treated animals were similar to the control animals). It should be noted, that the experiment described above was a single experiment with a limited number of animals (four or five per group). The data obtained in these initial experiments lead us to a more extensive study, using a preferred route of drug administration—i.e., intranasal administration.



Intranasal Administration of [St-Nle^{17}]VIP Protects Against Alzheimer-Related Retardation of Learning and Memory. As [St-Nle^{17}]VIP was originally designed to cross lipophilic barriers (32), we also tested the possibility of intranasal administration of the potent VIP analogue as a route of drug administration. Initially, penetration of ^{125}I-[St-Nle^{17}]VIP into the brain following intranasal administration was evaluated. Fig. 3 demonstrates incorporation of radiolabeled [St-Nle^{17}]VIP into various organs of the rat body 15 min after administration. Tissues enriched with [St-Nle^{17}]VIP were liver and frontal cerebral cortex (Fig. 3*A*). Counts per weight probably represent the VIP analogue in tissue, as the amount found in arterial blood (obtained from the orbital sinus) was at the most 10-fold greater than that detected within brain parts—i.e., cortex, hypothalamus, and brainstem. To assess for

FIG. 1. (*A*) Treatment with a fragment (amino acids 25–35) of the β-amyloid peptide produced neuronal cell death. Rat cerebral cortical cell cultures were treated with the β-amyloid peptide (amino acids 25–35) for 5 days. The β-amyloid peptide fragment was dissolved in water (to a final concentration of 2.5 mM). Each value is the mean ± SEM from four dishes. (*B*) [St-Nle^{17}]VIP protected neurons from the toxic effect of the β-amyloid peptide fragment. Increasing doses of [St-Nle^{17}]VIP (SNV) were added together with 25 μM β-amyloid peptide (amino acids 25–35) to dissociated cerebral cortical cells 9 days after plating of neurons. Parallel cultures were treated similarly with VIP in the presence of the β-amyloid peptide. Duration of treatment was 5 days with no change of medium. At the conclusion of the treatment period, neurons were identified immunocytochemically with antisera to neuron-specific enolase and counted. Each value is the mean ± SEM from four dishes. (*C*) [St-Nle^{17}]VIP protected neurons from naturally occurring cell death. Increasing doses of [St-Nle^{17}]VIP (SNV) were added to dissociated cerebral cortical cells 9 days after plating of neurons. Parallel cultures were treated similarly with VIP. Duration of treatment was 5 days with no change of medium. At the conclusion of the treatment period, neurons were identified immunocytochemically with antisera to neuron-specific enolase and counted. Each value is the mean ± SEM from four dishes.

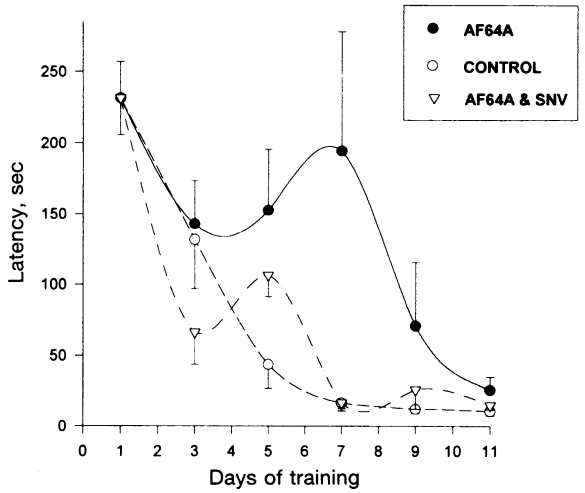


FIG. 2. [St-Nle[17]]VIP prevented learning deficiencies induced by cholinergic blockade. Thirteen male rats (Wistar; 250–300 g) were injected (i.c.v.); five rats served as controls and received an injection of saline (2 μl per side), eight other animals received injections of AF64A (3 nmol per 2 μl per side; ref. 9). Animals were stereotaxically implanted after injection to allow drug application and allowed to recover for a week. For behavioral testing, rats were placed in a circular pool equipped with a clear Plexiglas column, with a platform reaching just below the surface of the water. The eight animals injected with AF64A were divided into two equal groups and were thereafter injected daily (i.c.v.) with either saline or 100 ng of [St-Nle[17]]VIP (SNV) in saline 4 hr before behavioral testing. The five control rats (see above) were injected with saline. After 7 days of injections, behavioral assays were conducted for 14 additional days on both control and test animals. The latency of reaching the platform was recorded for each rat. Results are means ± SEM.

the integrity of the incorporated VIP analogue, radioactive tissue supernatants were subjected to HPLC analysis (30–32). Fig. 3*B* depicts a radioactive peak eluting from the HPLC column in fraction 30, corresponding to intact [St-Nle[17]]VIP. These data suggested that most of the injected [St-Nle[17]]VIP recovered in the brainstem supernatant remained undegraded 15 min after drug treatment. In parallel experiments, [St-Nle[17]]VIP was evaluated 30 min after topical administration. Results have demonstrated that 10% of the radioactive [St-Nle[17]]VIP remained intact in all tissues examined (32).

Administration of [St-Nle[17]]VIP intranasally to AF64A-treated animals significantly improved their performance in the Morris water maze in comparison to animals treated with AF64A alone (Fig. 4). AF64A animals treated with the VIP analogue (10 or 70 μg per day per animal) learned to reach the submerged platform that was rotated in the pool every day after 5 training days ($P < 0.05$), while AF64A animals treated with vehicle exhibited similar behavior only after 7 days of training. These results indicated an increase in learning and memory capacities associated with [St-Nle[17]]VIP (Fig. 4*A*). Similar results were obtained in other models exhibiting learning impairments, associated with aged rats or animals treated with VIP antagonists. Animals treated with AF64A and [St-Nle[17]]VIP exhibited a behavioral pattern identical to that of vehicle-treated controls. Treatment with [St-Nle[17]]VIP alone in control animals did not result in improvement of learning and memory (Fig. 4*B*). Fig. 4*C* depicts the results of a probe trial that assessed spatial memory. After 9 days of training and testing, the platform was removed and on day 10 the animals were subjected to swimming in a pool without the platform. It was apparent from the probe trial that there was a dose-dependent effect of [St-Nle[17]]VIP on spatial memory, as the time spent by the rats in the quadrant of the pool where the platform used to be was significantly increased in the VIP analogue-treated rats. The effect of [St-Nle[17]]VIP on spatial

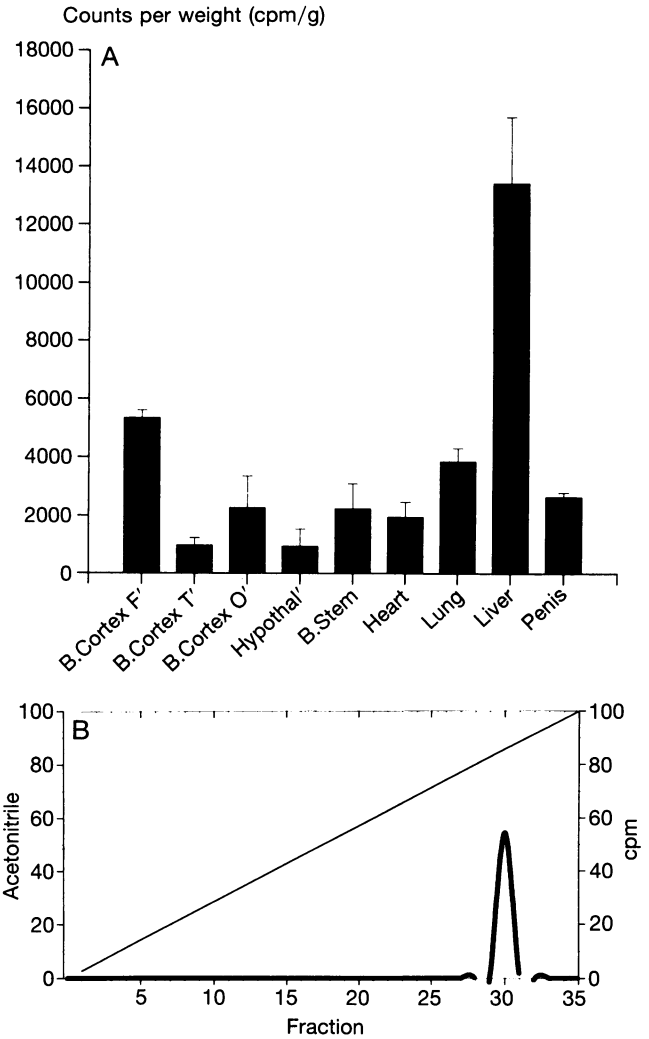


FIG. 3. Intranasally applied [St-Nle[17]]VIP reached the brain. (*A*) [125]I-[St-Nle[17]]VIP distribution after intranasal administration. The radioiodinated VIP analogue (3,458,794 cpm per 2 μl per rat) was applied intranasally to rats (250–300 g). Animals were sacrificed 15 min after drug administration and tissue samples were weighed and assayed for radioactivity in a γ-counter. B, brain; F', frontal; T', temporal; O', occipital. (*B*) Intact [St-Nle[17]]VIP reached the brain after intranasal administration. Radioactive tissue samples (e.g., brainstem containing 150 cpm per sample) were homogenized and subjected to centrifugation, and supernatants were analyzed by HPLC fractionation against [St-Nle[17]]VIP as a marker (eluting with an acetonitrile gradient at fraction 30). Samples were monitored for radioactivity in a γ-counter.

memory was apparent both in control rats ($P < 0.05$) and in AF64A-treated animals ($P < 0.007$). To evaluate for possible motor deficits that could impair performance unrelated to memory, on day 11 of testing the animals were allowed to swim to an exposed platform and, as evidenced in Fig. 4*A*, no significant difference was observed among the various treatment groups.

DISCUSSION

The present studies suggest that lipophilic analogues of VIP may be effective in the treatment of learning and memory disabilities due to pathological conditions related to neuronal deficits associated with Alzheimer disease. This finding may be of broader significance as, apart from Alzheimer disease, a variety of diseases and neurological deficiencies as well as normal aging may bring about degeneration of neuronal cells.

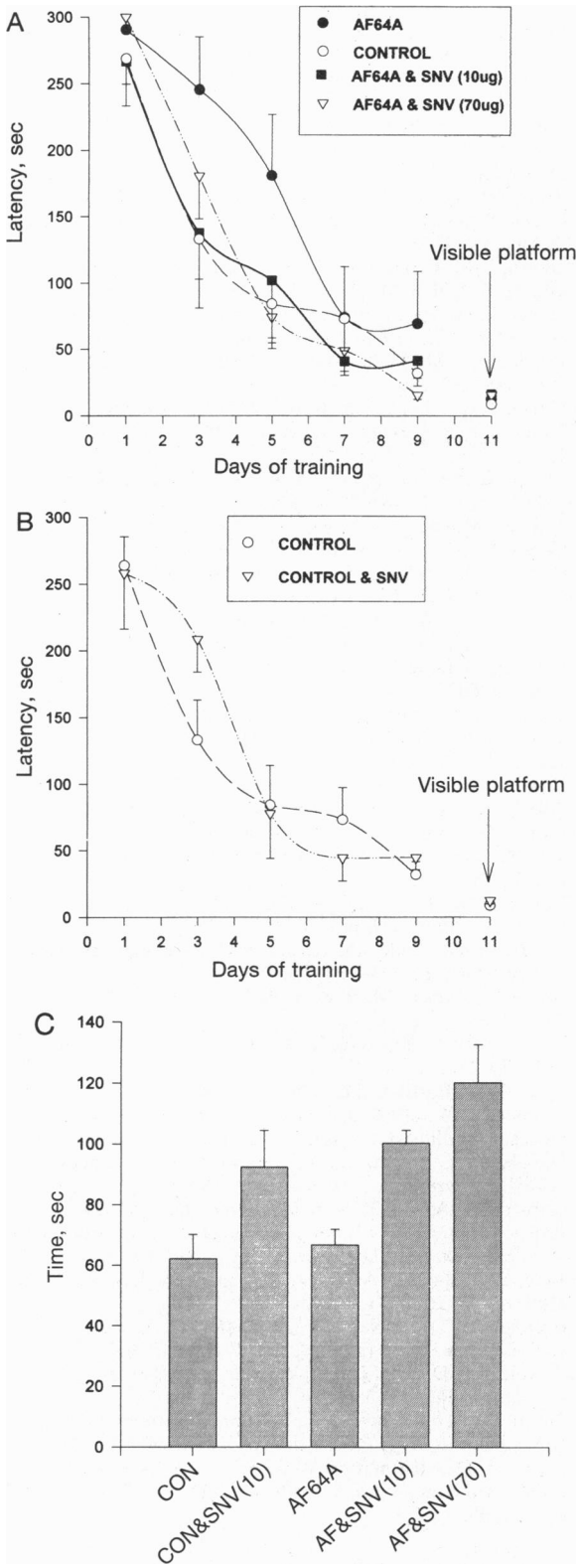


FIG. 4. Intranasally applied [St-Nle[17]]VIP prevented learning deficiencies induced by cholinergic blockade. Thirty male rats were treated with AF64A (as in Fig. 2) and 20 male rats were similarly treated with saline. Ten days after AF64A administration, 20 animals received daily nasal application of [St-Nle[17]]VIP dissolved in 10% sefsol/40% isopropanol. In 10 animals, the concentration of the peptide was 70 μg per 40 μl (20 μl administered through each nostril), and in another 10 animals the concentration was 10 μg per 40 μl per animal. Control animals (10 animals treated with saline instead of AF64A) received vehicle only and 10 control animals (treated with saline instead of AF64A) received 10 μg of [St-Nle[17]]VIP. After 7 days of drug administration, behavioral assays were conducted for an additional 11 days. Drugs were applied by nasal administration 1 hr before testing. The latency of reaching the platform was recorded for each rat (in seconds) as in Fig. 2. Experiments were repeated three times. (*A*) Comparison between control animals (saline treated) and AF64A-treated animals receiving [St-Nle[17]]VIP (SNV) at two different doses. (*B*) Comparison between control animals (saline treated) receiving intranasal administration of vehicle and control animals receiving 10 μg of SNV per rat. (*C*) On day 10 of testing, the platform was removed and a spatial probe test was performed. The animals were allowed to swim for 300 sec and the time spent by the animal at the platform quadrant was recorded. CON, sham operated animals administered vehicle only; CON&SNV (10), as above, administered 10 μg of [St-Nle[17]]VIP each; AF64A, animals administered AF64A and vehicle only; AF&SNV (10), as above, administered 10 μg of [St-Nle[17]]VIP each; AF&SNV (70), as above, administered 70 μg of [St-Nle[17]]VIP each.

With normal aging, there is a dramatic reduction in VIP mRNA in the cerebral cortex (22). Furthermore, a significant reduction of VIP immunoreactivity has been observed in the cerebral cortex, especially in the insular and angulate cortex of Alzheimer patients (29), although there is conflicting evidence concerning the role of VIP in dementia. One possible clue to VIP's role in dementia may be related to the involvement of this peptide with sleep regulation. It has recently been suggested that memory loss in Alzheimer disease correlates with rapid eye movement (REM) sleep deficits or malfunction (43). It has also been shown that VIP induces REM sleep (44) and waking induces accumulation of VIP in the cerebrospinal fluid, which in turn is involved in the production of REM sleep (45). The deficits in REM sleep apparent in Alzheimer disease are consistent with an impairment of VIP action.

The experiments described have shown that treatment with a neurotrophic VIP analogue had significant neuroprotective and functional benefits in several models which exhibited Alzheimer-related deficits. [St-Nle[17]]VIP had the remarkable property that it entered the brain intact after intranasal administration. This property greatly enhances [St-Nle[17]]VIP therapeutic potential, as a major obstacle in the use of any neurotrophic substance is the challenge of crossing the blood–brain barrier. These data are consistent with previous observations indicating that topically applied [St-Nle[17]]VIP exhibits stability and biological activity in noninvasive impotence treatment (32).

The mechanism through which [St-Nle[17]]VIP exerted neuroprotective effects is not yet clear. [St-Nle[17]]VIP has been shown to differentiate between two classes of VIP receptors in the central nervous system (34), preferring the high-affinity site and not recognizing the receptor associated with adenylate cyclase activation (42). The neurotrophic activity associated with VIP is apparently mediated through high-affinity receptors on glial cells (34, 46, 47) that are linked to mobilization of calcium (48) and the release of survival-promoting substances (46, 47). Our working hypothesis is that the neuroprotective action of VIP apparent in the Alzheimer models presented here is mediated through these glia-derived molecules. We further speculate that interference with the action of these endogenous molecules is a part of the etiology of Alzheimer disease. The behavioral model that included the cholinotoxin AF64A is known to induce neuronal cell loss (9–11), and specific neuronal cell loss has been associated with learning impairments (e.g., see ref. 39). Taken together with the ability of [St-Nle[17]]VIP to protect against neurotoxicity, one mechanism by which VIP analogues protect against learning and memory deficits may be via inhibition of accelerated neuronal cell death. The probe trial results suggest an influence of [St-Nle[17]]VIP on spatial memory and not an effect on motor activity; indeed, the time spent by the AF64A plus [St-Nle[17]]VIP-treated animals in the vicinity of the presumptive platform was significantly greater than that spent by the

AF64A-treated animals. In addition, when the animals of the various experimental groups were allowed to swim to an exposed platform, no differences were observed, indicating no motor deficits. In all tests performed, the effect of the AF64A was not apparent after 10 days of training compared to vehicle-treated control animals, suggesting recovery. In the probe trial, an influence of [St-Nle^{17}]VIP on the control animals was apparent. In contrast, an influence of [St-Nle^{17}]VIP on the latency of reaching the platform in control animals was not seen (Fig. 4*B*), emphasizing the significance of the additional probe trial, which picked up subtle differences (Fig. 4*C*). The effect of [St-Nle^{17}]VIP on control animals may be mediated by maintenance of neuronal survival and activity.

Regardless of the molecular mechanism by which VIP analogues ameliorate Alzheimer-related dysfunctions, the emerging possibilities of genetic counseling (49) and early detection with the advent of positron emission tomography (12, 13) may allow preventative and symptom-alleviating treatment of neurodegeneration with [St-Nle^{17}]VIP.

This paper was written in part while Prof. Illana Gozes was a Scholar-in-Residence at the Fogarty International Center for Advanced Studies in the Health Sciences, National Institutes of Health. We thank Dr. Haim Leder for the initial sample of AF64A. We thank Michal Bechar for her excellent collaboration and participation in the behavioral assays. This research is supported in part by Fujimoto Corp. and in part by the U.S.–Israel Binational Science Foundation.

1. Shapira, J. (1994) *J. Gerontol. Nurs.* **20,** 4–9.
2. Brumback, R. A. & Leech, R. W. (1994) *J. Okla. State Med. Assoc.* **87,** 103–111.
3. Kowall, N. W., Beal, M. F., Buscigilio, J., Duffy, L. K. & Yankner, B. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7247–7251.
4. Selkoe, D. J. (1993) *Trends Neurosci.* **16,** 403–409.
5. Schmechel, D. E., Saunders, A. M., Strittmatter, W. J., Crain, B. J., Hulette, C. M., Joo, S. H., Pericak-Vance, M. A., Goldgaber, D. & Roses, A. D. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 9649–9653.
6. Koliatsos, V. E., Price, D. L., Clatterbuck, R. E., Markowska, A. L., Olton, D. S. & Wilcox, B. J. (1993) *Ann. N.Y. Acad. Sci.* **695,** 292–299.
7. Price, D. L., Koliatsos, V. E., Sisodia, S. S., Koo, E. H., Martin, L. J., Walker, L. C., Applegate, M. D. & Cork, L. C. (1991) *Clin. Neuropharmacol.* **14,** Suppl. 1, S9–14.
8. Nitsch, R. M., Slack, B. E., Wurtman, R. J. & Growdon, J. H. (1992) *Science* **258,** 304–307.
9. Fisher, A., Brandeis, R., Pittel, Z., Karton, I., Sapir, M., Dachir, S., Levy, A. & Heldman, E. (1989) *Neurosci. Lett.* **102,** 325–331.
10. Gaspar, E., Heeringa, M., Markel, E., Luitan, P. G. M. & Nyakas, C. (1991) *Brain Res. Bull.* **28,** 65–71.
11. Dong, X. W., Hanin, I. & Loren, S. A. (1994) *Brain Res. Bull.* **35,** 217–220.
12. Nordberg, A., Lilja, A., Lundqvist, H., Hartvig, P., Amberla, K., Viitanen, M., Warpman, U., Johansson, M., Hellstrom-Lindahl, E., Bjurling, P., Fasth, K. J., Langstrom, B. & Windblad, B. (1992) *Neurobiol. Aging* **13,** 747–758.
13. Nordberg, A. (1993) *Acta Neurol. Scand. Suppl.* **149,** 62–65.
14. Said, S. I. & Mutt, V. (1988) *Ann. N.Y. Acad. Sci.* **527,** 1–691.
15. Gozes, I. & Brenneman, D. E. (1989) *Mol. Neurobiol.* **3,** 201–236.
16. Gozes, I. & Brenneman, D. E. (1993) *J. Mol. Neurosci.* **4,** 1–9.
17. Brenneman, D. E. & Eiden, L. E. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 1159–1162.
18. Brenneman, D. E., Westbrook, G. L., Fitzgerald, S. P., Ennist, D. L., Elkins, K. L., Ruff, M. R. & Pert, C. B. (1988) *Nature (London)* **335,** 639–642.
19. Gozes, I., Shani, Y. & Rostene, W. H. (1987) *Mol. Brain Res.* **2,** 137–148.
20. Baldino, F., Jr., Fitzpatrick-McElligott, S., Gozes, I. & Card, J. P. (1989) *J. Mol. Neurosci.* **1,** 199–207.
21. Dussaillant, M., Sarrieau, A., Gozes, I., Berod, A. & Rostene, W. H. (1992) *Neuroscience* **50,** 519–530.
22. Gozes, I., Shachter, P., Shani, Y. & Giladi, E. (1988) *Neuroendocrinology* **47,** 27–31.
23. Glowa, J. R., Panlilio, L. V., Brenneman, D. E., Gozes, I., Fridkin, M. & Hill, J. M. (1992) *Brain Res.* **570,** 49–53.
24. Gozes, I., Glowa, J., Brenneman, D. E., McCune, S. K., Lee, E. & Westphal, H. (1993) *J. Mol. Neurosci.* **4,** 185–193.
25. Lundberg, J. M., Hedlund, B. & Bartfai, T. (1982) *Nature (London)* **295,** 147–149.
26. Hedlund, B., Abens, J. & Bartfai, T. (1983) *Science* **220,** 519–521.
27. Eckenstein, F. & Baughman, R. W. (1984) *Nature (London)* **309,** 153–155.
28. Masuo, Y., Matsumoto, Y., Tokito, F., Tsuda, M. & Fujino, M. (1993) *Brain Res.* **611,** 207–215.
29. Arai, H., Moroji, T. & Koska, K. (1984) *Neurosci. Lett.* **52,** 73–78.
30. Gozes, I. & Fridkin, M. (1992) *J. Clin. Invest.* **90,** 810–814.
31. Gozes, I. & Fridkin, M. (1993) in *Peptide Chemistry 1992,* ed. Yanaihara, N. (ESCOM, Leiden, The Netherlands), pp. 442–445.
32. Gozes, I., Reshef, A., Salah, D., Rubinraut, S. & Fridkin, M. (1994) *Endocrinology* **134,** 2121–2125.
33. Forsythe, I. D. & Westbrook, G. L. (1988) *J. Physiol. (London)* **396,** 515–533.
34. Gozes, I., McCune, S. K., Jacobson, L., Warren, D., Moody, T. W., Fridkin, M. & Brenneman, D. E. (1991) *J. Pharmacol. Exp. Ther.* **257,** 959–966.
35. Romijn, H. J., Habets, A. M. M. C., Mud, M. T. & Wolters, P. S. (1982) *Dev. Brain Res.* **2,** 583–589.
36. Lilling, G., Wollman, Y., Goldstein, M. N., Rubinraut, S., Fridkin, M., Brenneman, D. E. & Gozes, I. (1995) *J. Mol. Neurosci.* **4,** 231–239.
37. Morris, R. G., Garrud, P., Rawlins, J. N. P. & O'Keefe, J. (1982) *Nature (London)* **297,** 681–683.
38. Morris, R. G., Anderson, E., Lynch, G. S. & Baudry, M. (1986) *Nature (London)* **319,** 774–776.
39. Moser, E., Moser, M.-B. & Andersen, P. (1993) *J. Neurosci.* **13,** 3916–3923.
40. Yankner, B. A., Duffy, I. K. & Kirschner, D. A. (1990) *Science* **250,** 279–282.
41. Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G. & Cotman, C. W. (1993) *J. Neurosci.* **13,** 1676–1687.
42. Gozes, I., Lilling, G., Glazer, R., Ticher, A., Ashkenazi, I. E., Davidson, A., Rubinraut, S., Fridkin, M. & Brenneman, D. E. (1995) *J. Pharmacol. Exp. Ther.* **273,** 161–167.
43. Christos, G. A. (1993) *Med. Hypotheses* **41,** 435–439.
44. Prospero-Garcia, O., Jimenez-Anquiano, A. & Drucker-Colin, R. (1993) *Neuropsychopharmacology* **8,** 387–390.
45. Jimenez-Anquiano, A., Baez-Saldana, A. & Drucker-Colin, R. (1993) *Brain Res.* **631,** 345–348.
46. Brenneman, D. E., Neale, E. A., Foster, G. A., d'Autremont, S. & Westbrook, G. L. (1987) *J. Cell Biol.* **104,** 1603–1610.
47. Brenneman, D. E., Nicol, T., Warren, D. & Bowers, L. M. (1990) *J. Neurosci. Res.* **25,** 386–394.
48. Fatatis, A., Holtzclaw, L. A., Avidor, R., Brenneman, D. E. & Russell, J. T. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 2036–2040.
49. Roses, A. D., Strittmatter, W. J., Pericak-Vance, M. A., Corder, E. H., Saunders, A. M. & Schmechel, D. E. (1994) *Lancet* **343,** 1564–1565.